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## INTRODUCTION

Understanding normal biology and its pathological deviations requires comprehending the form in which the different tissue components work together in their native tissue context. In particular, it requires studying cell-to-cell and cell-to-environment interactions, which tend to be highly variable between different tissue parts. This information can only be acquired by studying tissue in a state as similar to its native context as possible.

Contextual information, which is important for understanding normal tissue behavior, becomes essential when studying Breast Cancer, since heterogeneity and three-dimensionality are at the very base of cancer initiation and clonal progression. However, many analytical procedures in biology start by taking the element under study (RNA, DNA, etc) outside its native context. By doing so, an accurate measure of that element is achieved, but priceless information on how that element is related to its environment is lost. Other methods based on immunohistochemical staining of thin tissue sections for visual observation at the conventional microscope preserve only partial contextual information, since they provide a flat -two dimensional- view of a three-dimensional object.

Our goal is to develop a three-dimensional computer-based quantitative microscopy platform to be applied to simultaneous morphological and molecular studies of both normal and neoplastic mammary tissue. Using our system, we will be able to reconstruct relevant microscopic tissue structures (e.g. mammary ducts, lobules, lymph nodes, tumor masses, etc.) from consecutive thin tissue sections. Then, using the 3D virtual reconstruction, it will be possible to perform quantitative morphological measurements combined with directed -structure defined- high-resolution analysis of molecular events.

To show the use of our system, we will study the combined role of Estrogen and Progesterone Receptors (ER & PR) in mammary gland development. We will quantify cell-to-cell the levels of ER and PR in different parts of the mammary gland, at different time points of the development of the gland.

## BODY

Our accomplishments during the first year (08/01/00-07/31/01) of the project will be described following the Tasks enumerated in the approved proposal. The tasks are listed below, and the sub-tasks corresponding to the first funding year have been underlined. The text has references to the numbered tasks (e.g. see *Task 1.1*) where the fulfillment of the tasks is explained. Before starting describing them, it has to be noted that most of the goals have been amply fulfilled, including some that were scheduled for the second year, even though the effective start date of the work was 10/01/01, i.e. two months after the administrative start date. This has been possible in part thanks to the liberality of the contract officers, which allowed us to join the budgetary efforts of this and our other grant, "*Mechanisms of Intraductal Tumor Spread*" (DAMD17-00-1-0227), which goals for the first year partially overlapped with the goals of this grant.

**Task 1.** (*Months 1-12*) *Modify an existing microscopic imaging system for acquiring low magnification (1 pixel= 5  $\mu$ m) images of entire tissue sections and for tracing in 3D the ducts in the tissue specimen from a series of images of adjacent sections.*

1. Complete the existing JAVA based software for interactive marking and 3D virtual rendering of ducts so that it allows any branching pattern. (Months 1-6)
2. Develop a customized VRML viewer to allow visualization of the branching pattern and hyperlink it to the original images (Month 6-12)

**Task 2.** (*Months 6-30*) *Interface the existing acquisition and registration software with the JAVA application to allow revisiting of acquired slides for inspection and high-resolution acquisition of areas of interest. (Months 6-12)*

1. Integrate the JAVA application with the conventional fluorescence microscope (Months 6-12)
2. *Integrate the JAVA application with the confocal microscope (Month 12-18)*
3. *Integrate the high-resolution images in the VRML 3D representation of the mammary gland (Months 12-20)*
4. *Integrate the image analysis methods for nuclear segmentation with the JAVA application (Months 18-24)*
5. *Integrate the results of the segmentation with the VRML representation of the mammary gland. (Month 24-30)*

## ACOMPLISHMENTS

Under the combined budgetary efforts of this and grant DAMD17-00-1-0306, we have developed a client-server application, following the scheme shown in Figure 1. The server is a C application that runs on a computer (Dell Inspiron, running Solaris 7 for Intel) physically attached to an Axioplan (Zeiss Inc., Germany) microscope. The server provides access to the drivers that control the moving parts of the microscope: motorized scanning stage, excitation filter wheel and arc-lamp light blocking shutter (Ludl Electronic Products Ltd., Hawthorne, NY), as well as to the controller of the CCD Microlmager camera (Xillix Technologies Corp., Richmond, British Columbia, Canada). Besides giving access to basic operations, such as acquiring and storing images, setting the exposure time of the CCD or moving the stage and or the filter wheel, the server has been programmed to offer more complex functions. Some of them are automatic focusing of the microscope or acquisition of multiple-field of view images. To do this, the server receives the order and divides it into a set of simple actions. For instance, to

acquire a multiple field-of-view images, the server takes the coordinates of the vertices of the area to acquire and then automatically performs the sequence of stage movements and camera operations for first creating a focus map of the area and then acquiring a set of consecutive images that covers the area. The output is a mosaic-like image of the area. The server permits multi-color acquisition in fluorescence, by performing consecutive acquisition using different excitation filters.

The client (insofar referred as R3D2) is connected to the server through UNIX sockets, which are the standard for Internet-based communications. This means that it can send requests to the server from any computer internet-connected to the server. To obtain the maximum benefit from this fact, R3D2 has been written in JAVA (v.1.2), so that it can be executed on different computer platforms.

Figure 2 shows the R3D2's Graphical User Interface (GUI). The interface is divided in two distinguishable parts. One -rightmost vertical panel in Figure 2- provides connections to the server and allows the user to request its services through a user-friendly interface. The available actions are:

- *Connect*: Establishes a new connection with the server via an Internet socket. All the following options assume that this action has been successful.
- *Set Objective lens*: Our system does not have a motorized objective tower, and therefore this option does not effectively change the objective lens positioned in the light path. However, the user must update the lens when manually changing the lenses, because this information is required for some of the remaining options.
- *Change Filter*: Changes the excitation filter. This is needed when using fluorescence microscopy.
- *Exposure Time*: Sets the exposure time for the CCD camera (in ms.)
- *Set Origin*: This option moves the scanning stage of the microscope to its absolute mechanical origin of coordinates, relative to which all future movements and measurements are done. This is required to precisely locate the areas of the slides where images are taken, which is necessary for accurate revisiting of the slide and correct reconstruction of the tissue.
- *Acquire image*: Takes an image using the existing settings. R3D2 receives the image from the server and displays it, both complete -zoomed out- as well as partially -in their original resolution-. Only a small part of the full-resolution image can be displayed at full resolution. The area can be interactively determined by moving a window on the complete version of the image (**Figure 3**). Images can be saved both in ICS [Dean 90] and JPEG format. When images are saved as ICS, all the acquisition parameters (objective, filter, location of the image on the slide are, etc.) are stored in the ICS header file.

- *Autofocus*: Automatically focuses the microscope by taking a series of images at different positions in the Z axis (step size=0.5 microns) and determining the best focused image of the series. Blur due out of focus light reduces image contrast, which can be detected using several functions. Based on several comparisons described in the existing literature [Groen 85][Firestone 91][Santos 97], we selected an autocorrelation-based measurement introduced by Vollath [Vollath 87].
- *Open/Close Shutter*: Closes the arc lamp light blocking shutter, to reduce photobleaching due to unnecessary light exposure. This is useful when using fluorescence microscopy.
- *Scan*: Acquires multiple field of view images. The system displays a dialogue-panel (Figure 4) where the user can specify the filter(s) to be used (in fluorescence microscopy), exposure time(s) and the limits of the area to acquire. The limits can be defined by its coordinates (when known) or manually, by moving the microscope to the upper, lower, rightmost and leftmost points of the area. Figure 5 shows an example of an image of a complete tissue section.
- *Revisit Point*: When the user clicks on a point of a previously imaged section, the server moves the stage to that location on the slide and takes an image using the current values of objective, filter and exposure time.
- *Revisit Area*: When using the option, the user is prompted to draw a rectangle on the image of a section. Then a dialogue panel similar to the one used to acquire sections asks the user for the parameters of the acquisition(s). Then the system automatically acquires the area(s). This can be used to re-acquire areas using a different magnification and/or filter (when using fluorescence). This feature greatly simplifies some tasks, such as determining and acquiring areas of interest (e.g. sections of mammary ducts, blood vessels, tumor micro-invasions, etc.) on whole tissue sections. Using our system one can, for instance, acquire a whole-view of a section at low magnification (x2.5, x10), and then revisit as many areas as he/she wants for acquisition (e.g. all the ducts on a section of mammary gland) at higher magnification (x40, x63, x100). The process is shown in Figure 6. Multiple areas within a section can be background-acquired by the system without any human interaction. (**See Task 2.1**)

Besides providing access to the server functions. The second part of the interface (two left vertical panels in Figure 2) expands the system capabilities, by allowing creating and handling sets of related images, which we call *Cases*. A case can have multiple-color, multiple-magnification related images, easily accessible and meaningfully stored.

For us, a case is a sequence of low-magnification images of complete tissue sections taken from a tissue block, along with all the areas re-visited on them at higher resolution and/or with different filters. Finally the user can add information to the cases, by marking and/or delineating structures of interest and linking them within and between

consecutive sections (**See Task 1.1**). These are the types of marks and some actions that can be performed related to them:

- *Text*: Textual annotations to identify structures of interest for future study or to draw the attention of a different user.
- *Duct*: Identifies ductal structures. A section of a duct perpendicular to the sectioning plane is marked with a circle in the center lumen. Each circle has a unique number that identifies it. Its coordinates are stored in a data structure that contains information on all the ducts and their interconnections.
- *Connected Ducts*: Connects ductal marks. Within the same section, an elongated, longitudinal section of a duct, is normally marked with two *Duct* marks connected by a line. If both ends are located on different sections, the connection is displayed by changing the color of both *Duct* marks when the mouse travels over any one of them.
- *Shapes*: Delineates irregularly shaped ducts or other structures (e.g. tumor masses, lymph nodes, etc.).
- *Groups*: Groups series or *Shapes* corresponding to consecutive sections. The *Shapes* are grouped by sequentially clicking on them while the option to create *Groups* is on. To show grouping, the system changes the color of all the *Shapes* of the group every time the mouse moves into any one of them. *Groups* identify connected objects, such as sections of a tumor mass, lymph nodes, etc.
- *See Area*: Displays previously acquired images of Areas of interest, when the user clicks on the rectangle that locates them within their corresponding image section.

Before reconstructing a Case in 3D, all its sections must be registered. For that, we calculate the Rigid-Body Transform that gives the optimum rotation and translation between each pair of sections. The Transform is calculated from six pairs of points interactively marked by the user on each pair of images to be registered. Once the points have been marked, the software calculates the rotation and translation ( $\theta$ ,  $t_x$ ,  $t_y$ ) needed to minimize the sum of the squared distances between all six pairs of corresponding points. The results are stored in the ICS header file of the second image. When registering a Case, all sections are aligned with the first section of the case, either by directly registering them to the first section or by using already corrected coordinates of contiguous sections.

This method is very accurate when the pairs of points are spread all along the sections. Reasons for small errors are imprecise mouse interaction, stretching and/or compression of the tissue, and the fact that some structures used to select pairs of corresponding points might not be perpendicular to the sections. Using as many pairs of points as possible can average out these small errors. That is why, instead of using just four pairs of registration points –which is the minimum required for this method to work– we use six.

An important feature of R3D2 is that all Case-handling and marking functions can be used in parallel to the functions that request microscope actions. Therefore, acquiring a new section can be done in parallel to any other Case related function (e.g. registering already acquired sections or annotating the images). This is possible because we are using Solaris threads. Threads permit executing multiple parallel copies of a program without multiplying resource use. Each thread shares memory and other resources with other threads. R3D2 runs on a main thread and when a microscope-based operation is selected, it launches a new thread that runs on the same memory space as the main one. Thus, the client can accept handling and marking operations on the main thread while a microscope-based operation is executed at the new one. This also guarantees that, in the case of a microscope failure or a socket error, the system will not die abruptly, as only the thread working on the microscope will be affected.

The most innovative feature of our system consists on its ability to reconstruct the tissue structures by rendering the user markings in 3D (**See Task 1.1**). For that, we have used Java3D (v.1.2.1.) This is an Application Programming Interface (API) for 3D in Java. After asking the user for the range of sections to render, the system converts the coordinates of all the markings in that range of sections from two-dimensional to three-dimensional values. The section number and thickness, along with the distance between sections, determines the Z –depth- coordinate. Then a 3D scene is built using several geometric shapes to represent the different markings: spheres for sections of ducts perpendicular to the section, lines for connections, polygons for shapes, ... Once the Case is rendered (using another thread) it is displayed in a new window where the mouse can be used to rotate the scene, to zoom in or out or to translate the scene in the X and/or Y directions.

The 3D window includes a tool bar with options to select elements. Selecting elements one can obtain information about them, load the image(s) that contain that element or move the microscope to its location on the slide for re-imaging. That way, by just clicking on elements of the 3D scene, the user can both display and/or revisit areas of interest which could have been otherwise missed using a conventional 2D analysis (**See Task 1.2**). The window also has buttons to hide/show connections, shapes, areas (acquired at higher magnification) and the three-dimensional coordinate axis. There is also a button to reset the scene to the default view and combo boxes to change the scale in any of the three dimensions.

We will now illustrate the use of our system by showing the steps followed to image and reconstruct two different tissue blocks. These blocks were used to test the system. Both blocks have equal tissue origin –mouse mammary gland- and correspond to two different modalities of optical microscopy. The first block is an H&E stained, paraffin embedded gland, the second is a fluorescently counterstained, frozed gland.

#### **Tissue source**

Female BALB/c mice were obtained from Simonson (Gilroy, CA) and housed 4 per cage with chow and water *ad libitum* in a temperature and light controlled facility. Carbon dioxide inhalation was used to kill the animals in accordance with Association for Assessment and Accreditation of Laboratory Animal Care guidelines and institutional review and approval. The 3rd and 4th inguinal glands were removed for histology and whole mounts at 4, 6, 8 & 10 weeks.

### **Tissue preparation**

One gland (MP1, 10 weeks old) was paraffin-embedded, H&E stained. A second gland (MF1, 8 weeks old) was frozen and immersed in Evan's Blue, which stains both nuclear and cytoplasmic areas. Evan's Blue fluorescent emission is bright red, similar to Texas Red. Both glands were sectioned at 4  $\mu\text{m}$ , and placed on glass slides for microscopy and imaging.

### **Imaging of tissue sections**

Full tissue sections were imaged in bright field (MP1), or fluorescence (MF1), using R3D2's *Scan* option (See Figure 7). Sections were imaged at 10X, with a Fluar (0.5 na) objective lens (Zeiss, Wetzlar, Germany). In order to match the emission of Evan's Blue, a filter set for imaging Texas Red was used. To optimize memory use while keeping enough resolution for Histology, images were reduced by a factor of 4 in both X and Y directions, which gave us an effective 2.5X magnification, i.e. a sixteen fold reduction in image size. After image compression, the average image was 10Mbytes. This reduction was necessary to speed up image transmission from the server and display on the client GUI.

### **Creation of Cases**

Two cases were created which included all sections from each of the imaged glands. (See Figure 8). Cases were created empty, and images of the sections added as they were being acquired. MP1 had 24 sections, from approximately half of the mammary gland, while MF1 had 92 sections, which corresponded to a fully sectioned complete mammary gland.

### **Tissue Annotation**

All sections were manually annotated. We marked the centers of the ducts by placing a circular mark (R3D2's *Duct* tool) in the lumen -when the duct was perpendicular to the image plane- or with a line -when the duct was sectioned longitudinally-. Then we connected the markings within and between consecutive sections using the *Connect Ducts* option. We also delineated the lymph nodes using R3D2's *Shapes* option and grouped their consecutive sections using the *Group* options.

### **3D Reconstruction**

Figure 9 shows the rendering of the annotations done on the cases. The reconstruction is fully interactive in that by clicking on the markings one can: i) retrieve information (location, image statistics of the surrounding area); ii) display the area of the original image that contains that annotation or iii) automatically move the microscope to that location, if the appropriate slide is placed on the stage.

## **PROBLEMS**

The main problems faced so far are:

- The size of the images, when acquiring entire tissue sections even at low magnification (10x), is quite large (130 Mb. or more). This exhausts the computer memory when more than one full-section is displayed, even using a computer with 512 Mb of RAM memory, the reason being the high memory consumption of the Operating System and the JAVA Virtual Machine, which leave limited resources for the client requirements. In order to make the system work at a

reasonably speed without compromising its use, we introduced a compression factor which can be set by the user before acquiring a section. The user can choose between acquiring the image at full resolution (10X), half resolution (effective 5x) or at one fourth of the original resolution (effective 2.5X). Using the highest compression factor ( $\frac{1}{4}$ ) is recommended when imaging tissue sections larger than  $1 \text{ cm}^2$ . Smaller tissue sections can be acquired using half or full resolution. Consistency requires maintaining equal resolution for all sections of a case.

- Scanning whole sections is an unsupervised operation, which uses parameters previously defined by the user. The exposure time is one of these parameters. That value is set before starting scanning and does not change until the scanning is done. In fluorescence, this is a problem, since the effect of the preparation protocols (e.g. protein digestion, nuclear permeabilization in FISH or Immunohistochemistry, etc.) varies from cell type to cell type. Thus the concentration of fluorescent material that reaches its target, may it be the chromatin, a gene or a protein, varies between different parts of the tissue. Therefore, the exposure time that is appropriate for one part of the tissue may not be appropriate for another. That affects the quality of the images, since some areas tend to be either overexposed or underexposed. At the moment we manually selected an intermediate value that allows us to image dark areas without overexposing bright areas. This works in almost all cases, but renders images with varying contrast. To avoid that, we are developing an algorithm which, when activated by the user, will adjust the exposure time to the fluorescence intensity.
- An important problem is that, due to tissue complexity, image annotation of real tissue specimens is very time consuming. This is true, even when reconstructing fully sectioned mammary glands of young animals (6 weeks). In that specific case, the average number of ducts per section is 100, having all of them to be marked and connected to their corresponding ducts on the two contiguous sections. If the gland was fully sectioned at e.g. 4 microns, each case has an average of 100 sections, leaving us with 10000 markings and 40000 connections per case, which is absolutely impracticable. However, most of our studies scheduled for the two coming years don't require fully sectioning of glands, since a correct planning the sectioning will reduce the number of sections to approximately 20. There is also the immediate option of increasing the distance between sections, using only one every  $N$  sections for the reconstruction.  $N$  has to be low enough as to ensure that it is still possible to determine ductal continuity between adjacent sections. We are now in the process of determining the optimum value. Finally, we are working on algorithms to automate the detection of ducts. The options are to fully automatic detection and reconstruction the ducts, followed by interactive correction, or manual marking of an intermediate slide and then automatic 'growth' of the structures from that slide towards the contiguous sections.
- A final problem, related to the previous one, is the redundancy of information in the 3D reconstruction. Marking the same structure on consecutive sections sometimes leads to 3D objects with too much level of detail. This can complicate the interpretation of the results. We are working on the way to 'prune' the 3D reconstruction of the markings and create a skeletonized version of it.

**Task 3.** (Months 6-36) Use #1 & #2 to perform 3-D reconstruction maps of the distribution of ER and PR in the ductal tree and determine whether ER colocalizes with PR in mouse mammary epithelium during critical phases of mammary development.

1. Select the animals (20) to study, taken at different stages of development.  
Extract, fix and section the tissue as explained in the Methods Section (Months 6-12, 4 specimens; Months 12-24, 8 specimens, Months 24-36, 8 specimens)
2. Reconstruct the mammary gland using the software developed according Task 1. (Months 12-24, 5 specimens; Months 24-36, 15 sections)
3. Revisit areas of interest for high resolution imaging and detection/colocalization of Progesterone and Estrogen receptors) (Months 24-36, 20 sections) Integrate the information (Months 30-36)

For the reporting period, the only scheduled sub-task was selecting the glands that will be analyzed and reconstructed in the following two years. Given that the sample preparation time is relatively short (2-3 days) and has to be done immediately before the sections are imaged, we have delayed selecting the animals and preparing the samples until right before they are analyzed.

## **KEY RESEARCH ACCOMPLISHMENTS**

The first year of the grant was used to develop the microscopy and computer infrastructure that will be used in the following two years. In summary:

- We have developed a 3D computerized microscopy platform that allows image acquisition of entire tissue sections at low resolution, and then provides interactive tools for marking structures of interest (in our case mammary ducts, tumor masses, etc.). The system can reconstruct the annotations in 3D to provide a better understanding of tissue organization, which can be used to plan high-resolution analysis of areas of interest.
- The 3D reconstruction is linked to the microscope, to allow revisiting and re-acquisition of areas of interest at high magnification and multiple fluorescent channels (when using fluorescence microscopy).
- To test the system, we have successfully imaged and reconstructed two mammary glands, stained with different methods (H&E and fluorescence). By doing it we have achieved an understanding of the system that will help us to design the research goals proposed for the coming two years.

## REPORTABLE OUTCOMES

### Manuscripts:

- *"Recent advances in quantitative digital image analysis and applications in Breast Cancer"*. Ortiz de Solorzano C., Callahan D.E., Parvin B., Costes S., Barcellos-Hoff, M.H. Review paper submitted to *Microscopy Research and Technique*.
- *"Application of three-dimensional image analysis to the study of Breast Cancer"*. Ortiz de Solorzano C. Proceedings of the XX Congress of the Spanish Society of Pathology. pp. 253-257 (In Spanish), Pamplona, Spain July 1-5, 2001, 2001.
- *"A system for computer-based reconstruction of 3-dimensional structures from serial tissue sections: an application to the study of normal and neoplastic mammary gland biology"*. Fernandez-Gonzalez, R., Jones A., Garcia-Rodriguez E., Knowles D., Sudar D. Ortiz de Solorzano, C. Proceedings of Microscopy and Microanalysis'2001. Microscopy and Microanalysis 7(suppl. 2), pp. 964-5, 2001.

### Presentations:

- *Application of three-dimensional image analysis to the study of Breast Cancer*. XX Congress of the Spanish Society of Pathology. Pamplona, Spain July 1-5, 2001, 2001. Invited talk
- *A system for computer-based reconstruction of 3-dimensional structures from serial tissue sections: an application to the study of normal and neoplastic mammary gland biology*. Microscopy and Microanalysis'01, Long Beach, CA August 5<sup>th</sup>-9<sup>th</sup>, 2001. Platform presentation.

### Informatics:

- As described in the Body of the report and in the Reportable Outcomes sections, we have developed a three-dimensional computerized 3D microscopy platform (R3D2).

### Funding applied:

- *Segmentation of Mammary Gland Ductal Structure Using Geometric Methods*. P.I.'s Malladi R. and Ortiz de Solorzano C. Submitted to the LBNL Laboratory Directed Research and Development Program (LDRD) 2002, in the Strategic-Computational Sub-Program.

Employment or Research:

- Upon reception of the Award, I was offered, and accepted, a Scientist Position at the Life Sciences Division, Lawrence Berkeley National Laboratory of the University of California.
- The outstanding work of my Research Assistant (Rodrigo Fernandez Gonzalez) on the development of the microscopy and computing infrastructure has been instrumental in his acceptance to the joint UCSF-UC Berkeley Ph.D. program in Biomedical Engineering. He will continue working part-time on this project as Graduate Student Research Assistant.

## CONCLUSIONS

In summary, the goals for the reporting year have been fully accomplished. We have developed a three-dimensional computerized microscopy platform that allows imaging and 3D-reconstruction of tissue blocks to combine morphological (Histo-Pathology) and molecular (Immunohistochemistry) information. Our platform directs and simplifies image acquisition at different resolution levels and rationalizes image storage and retrieval.

Applying our system to the test cases described in this report has helped us understanding some practical limitations of the described methodology (e.g. high memory and storage needs for the data sets or high interactive burden when manually annotating tissue structure). Practical solutions have been proposed and implemented, and therefore the existing system is appropriate for the analysis planned for the following two years –studying the whole-gland distribution and interplay of Estrogen and Progesterone receptors in normal and neoplastic mammary gland development-. However, we will devote some limited resources to exploring further automation of the two most time-consuming tasks: image acquisition and tissue annotation. Imaging of whole sections can be sped up by using an automatic slide feeder attached to the microscope, so that all sections can be acquired without requiring manual interaction. Tissue annotation interaction can be greatly reduced by using existing image analysis algorithms for automatically detecting tissue structures (Ducts, lymph nodes) and connecting them between sections. By using them, interaction will be then limited to annotating one section of the case and correcting the results of the automatic detection.

The relevance of what we have achieved is significant, since our system can and will eventually be applied to a variety of studies, both molecular and clinical, which will greatly profit from the additional –third- dimension. Several Pathologists have expressed their interest in our system, arguing that it will improve the way they look at the samples and the way they handle and store ‘visual’ information. Full use of this system in a clinical setting requires further automation in the areas above mentioned, to provide additional information without a substantial increase of diagnostic time. Similarly, several colleagues in the Breast Cancer Research field have expressed their interest in our system, which would allow them to do whole sample or whole gland studies similar to the one proposed in this grant.

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## FIGURES

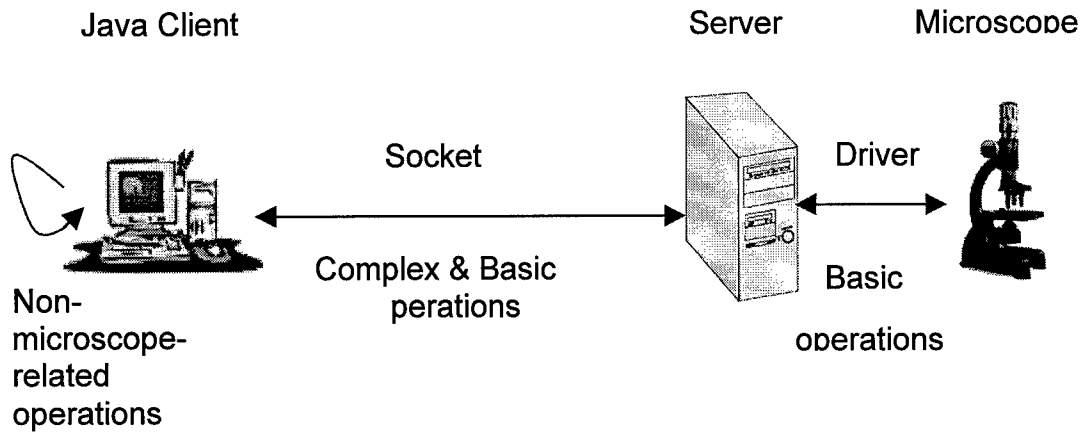


Figure 1. Description of the system

The application uses client-server architecture. The server runs on a computer physically attached to the microscope, and provides access to the microscope functions. The client is a JAVA application, which can run on any computer (no particular OS required) connected to the server through the Internet Protocol (IP). The communication between client and server uses UNIX sockets. The client provides user-friendly access to all the microscope functions offered by the server, and allows handling of sets of related images (Cases) for storage, annotation and 3D reconstruction of structures of interest.

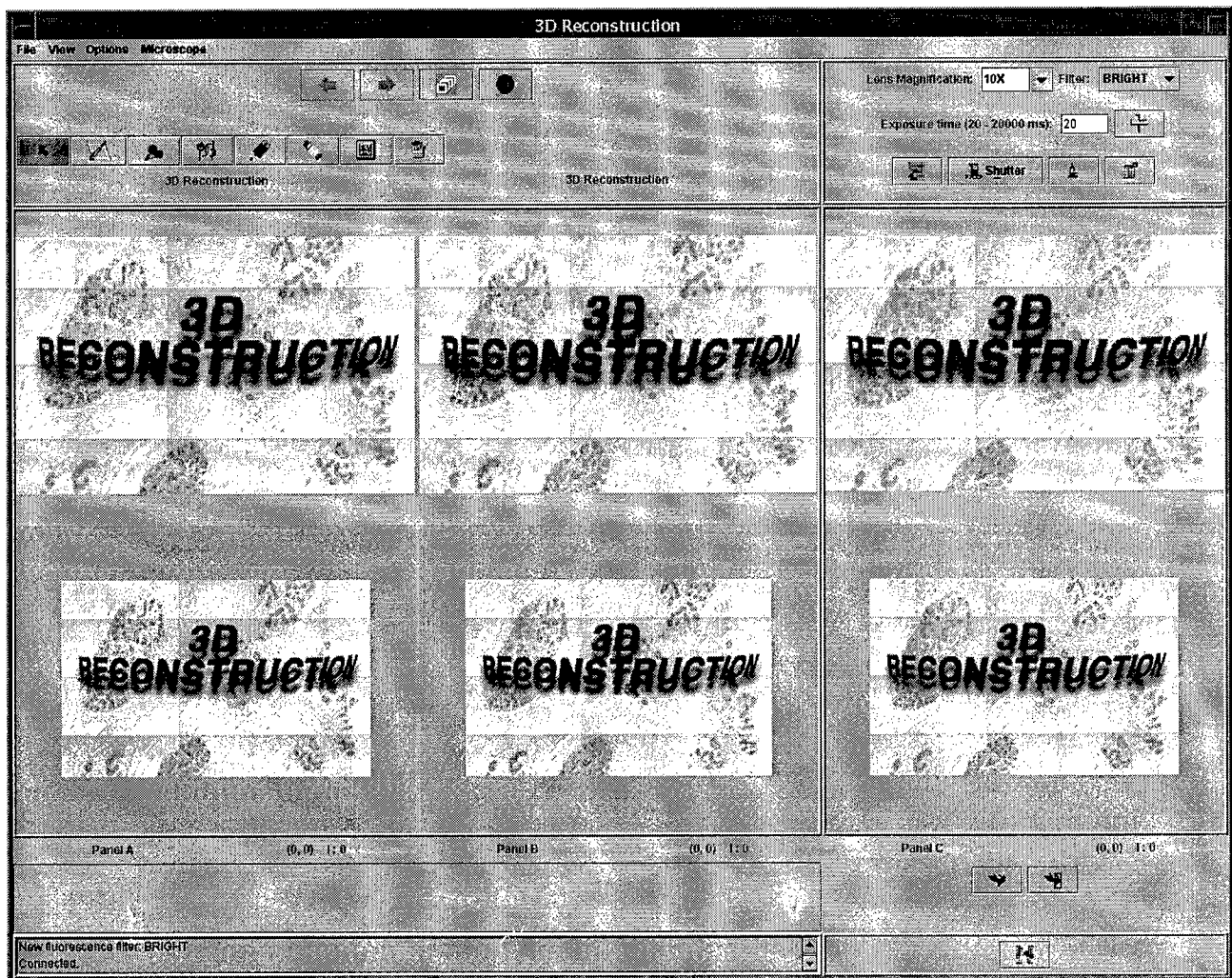


Figure 2. R3D2's Graphical User Interface.

The client application (R3D2) is a JAVA application. The GUI is divided in two main parts. The two leftmost panels are for displaying consecutive sections of a Case. It also provides the user with tools for registering the sections, annotating the images and connecting structures between sections (e.g. mammary ducts, tumor volumes), and reconstructing the annotated images in 3D. The rightmost panel gives access to the microscope related functions offered by the Server.

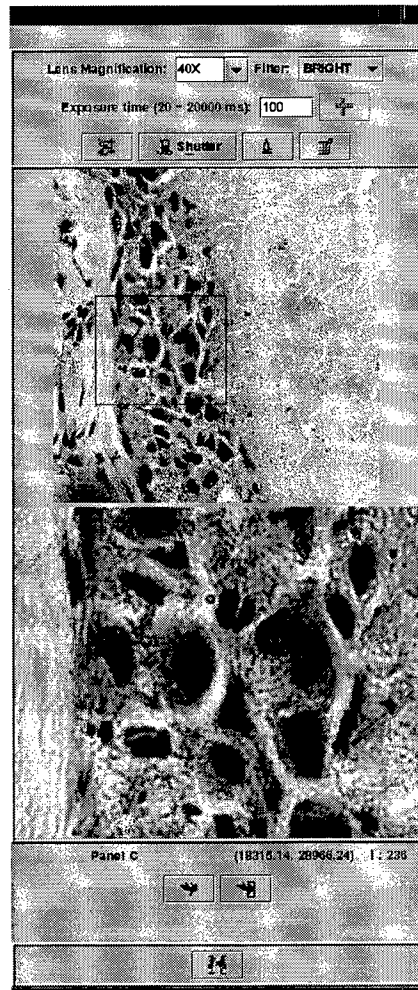
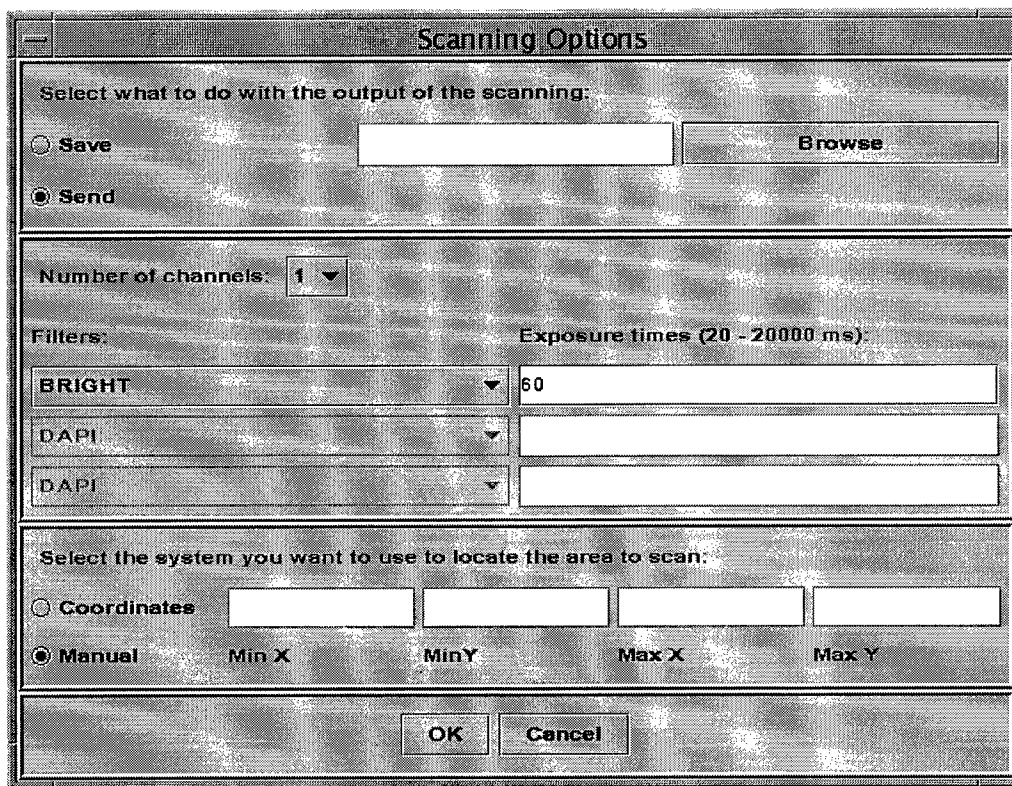


Figure 3. Acquisition of a single image

To acquire a single image, the user clicks on the “Acquire” button, after choosing the appropriate microscope settings: objective lens, excitation filter (in fluorescence) and exposure time of the CCD camera. The “Autofocus” option automatically finds the correct focus plane of the microscope before acquiring an image. The top panel contains the entire image (reduced to fit in the window), while the lower panel contains a zoomed version of the part of the original image selected by the rectangle on the top window. The rectangle can be manually moved to look at different parts of the image at its original –full- resolution.



**Scanning Options**

Select what to do with the output of the scanning:

☐ Save

☒ Send

---

Number of channels:  ▼

Filters: Exposure times (20 - 20000 ms):

BRIGHT ▼	<input type="text" value="60"/>
DAPI ▼	<input type="text"/>
DAPI ▼	<input type="text"/>

---

Select the system you want to use to locate the area to scan:

☐ Coordinates

☒ Manual Min X Min Y Max X Max Y

Figure 4. Panel for acquisition of whole-sections

To acquire multiple field-of-view images, the user fills out this form, which requests the name of the file where the image will be stored (if not only displayed), the number of scans –in fluorescence, and which filter and exposure time are to be used in each scan, and the coordinates of the limits of the area. The coordinates can be entered in the boxes, if known, or otherwise interactively defined at the microscope.

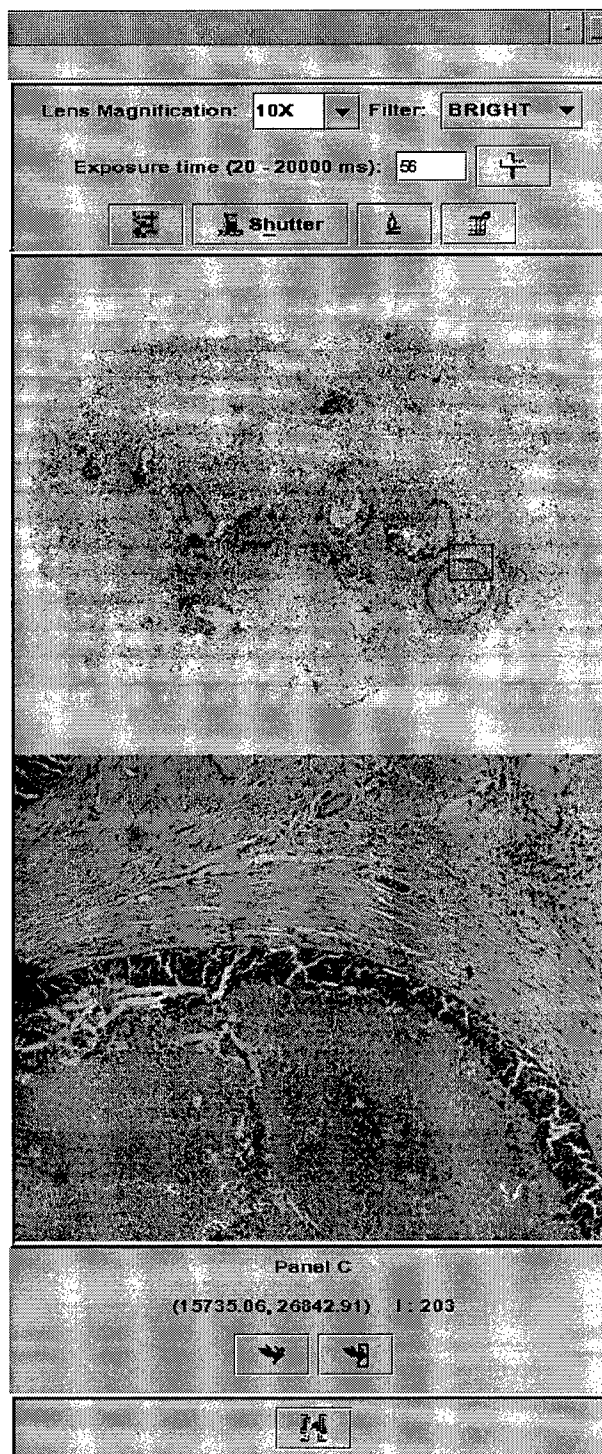


Figure 5. Whole section acquisition

This image shows the result of scanning a complete tissue section from a tissue block of a patient with Ductal Carcinoma In Situ of the Breast (DCIS), stained for Histology with Hematoxylin and Eosin. As for the individual images, the upper panel contains the entire section while the lower contains a zoomed sub-area of it.

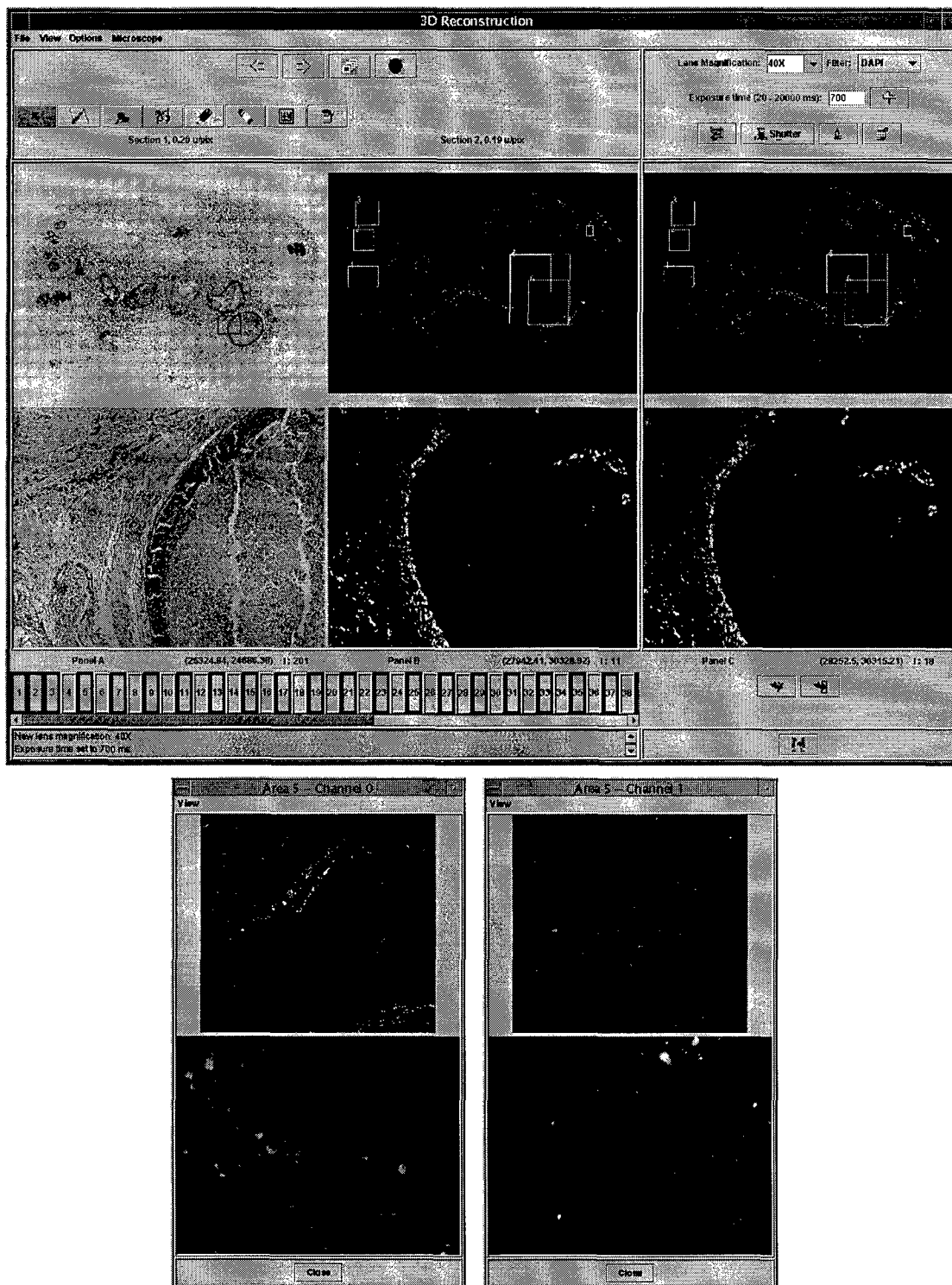


Figure 6. Revisiting of Areas of Interest

Top. R3D2 showing a case of a tissue block from a patient with Ductal Carcinoma In Situ of the Breast. The block was sectioned and stained with H&E (odd sections)

and DAPI –counterstain- and FISH to the erb-b2 gene. The interface shows two sections. The left section (the fluorescent one) shows several light (yellow) rectangles which correspond to areas which were revisited and reacquired at a higher resolution. By clicking on a rectangle, the user can display those areas in new panels, which show, in this case, a counterstained area (Bottom, left) and the image channel corresponding to the emission of the fluorochrome attached to the probe to the erb-b2 gene hybridized on the section (Bottom, right).

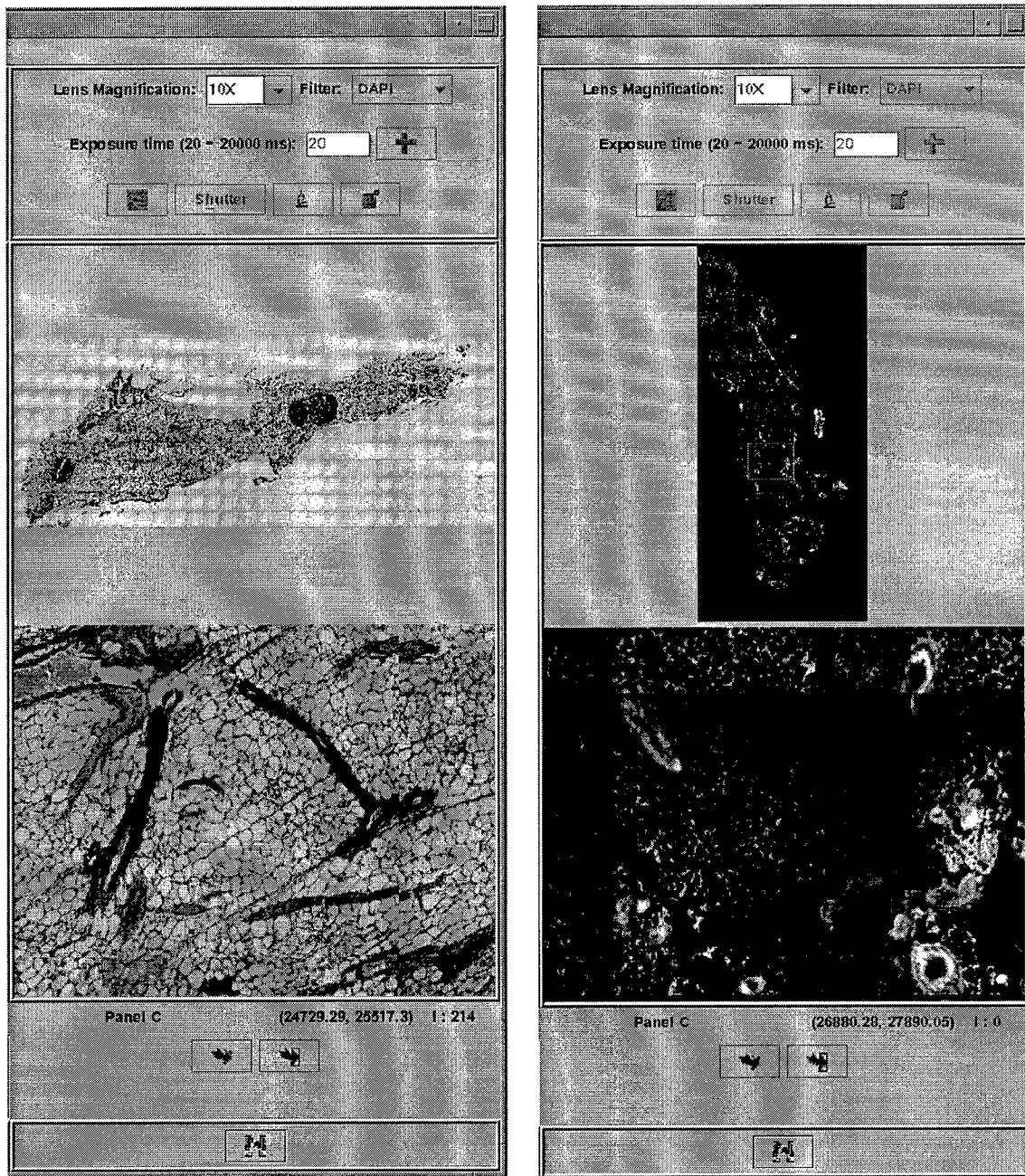
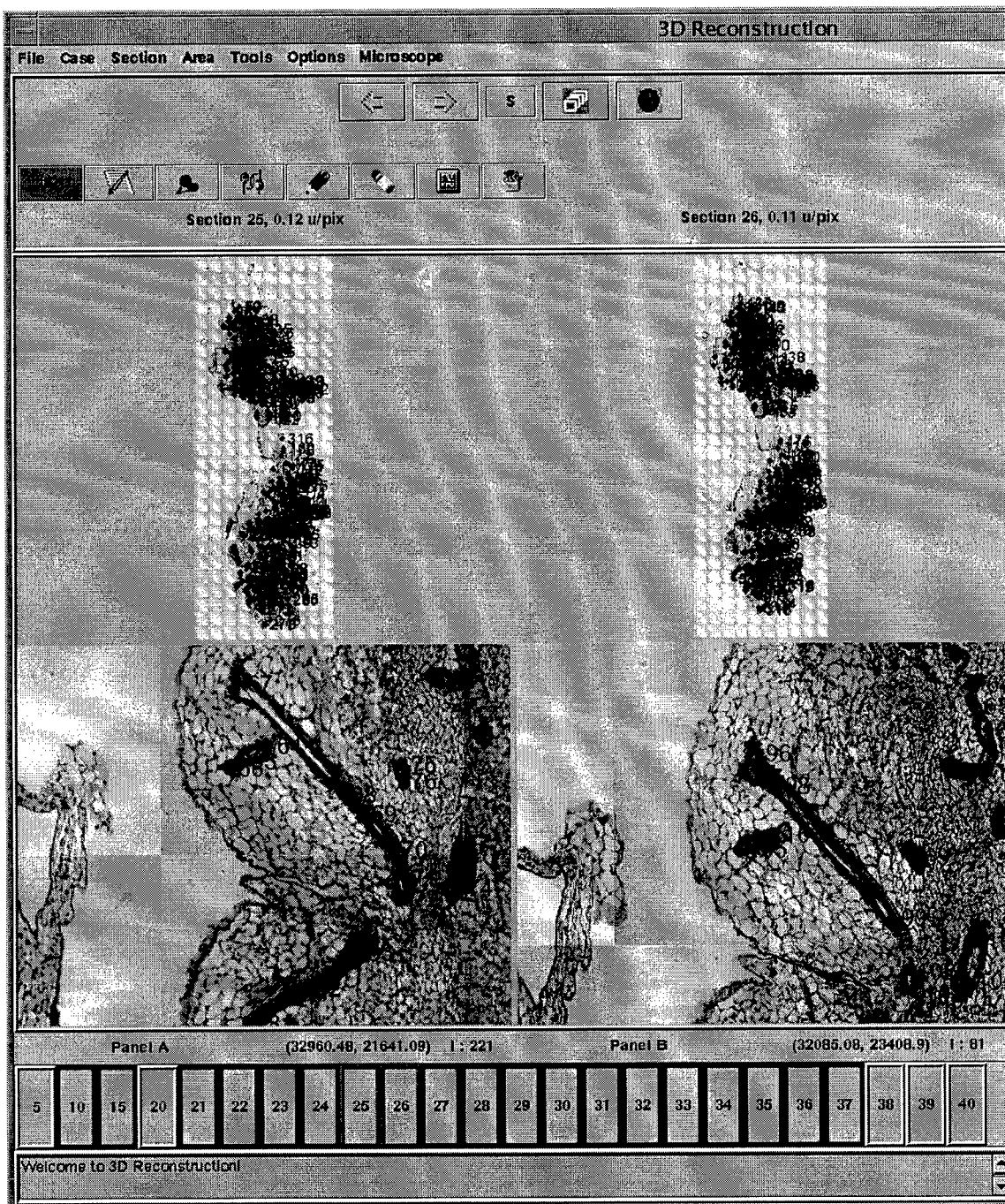
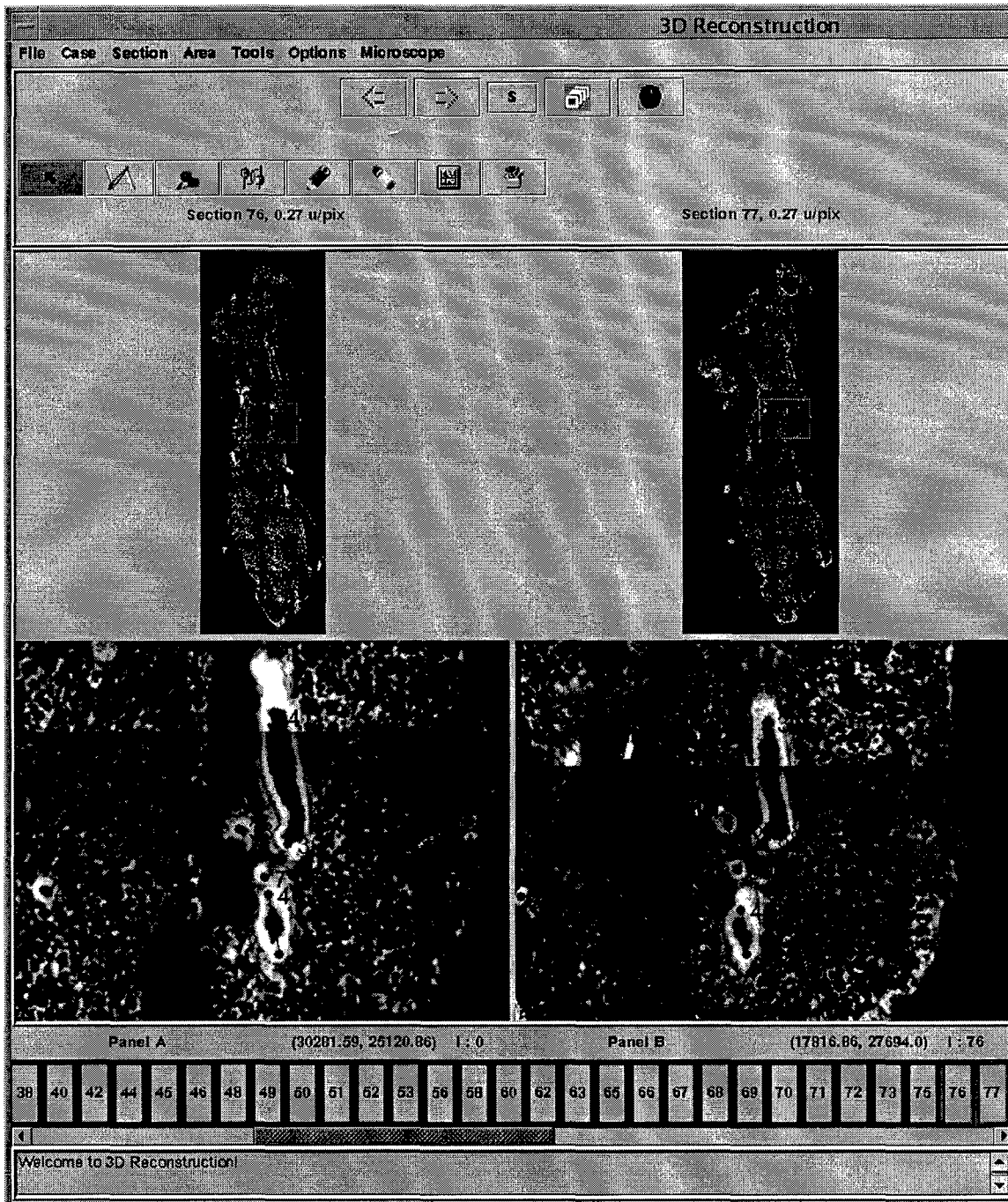


Figure 7. Tissue acquisition.

The image shows two sections from the cases used to test the systems. Left. Tissue section of an H&E stained mouse mammary gland. Right. Tissue section from a mouse mammary gland stained with Evan's Blue, which stains both nuclei and cytoplasm. Microscope focusing and image acquisition is completely automated, as described in the text.



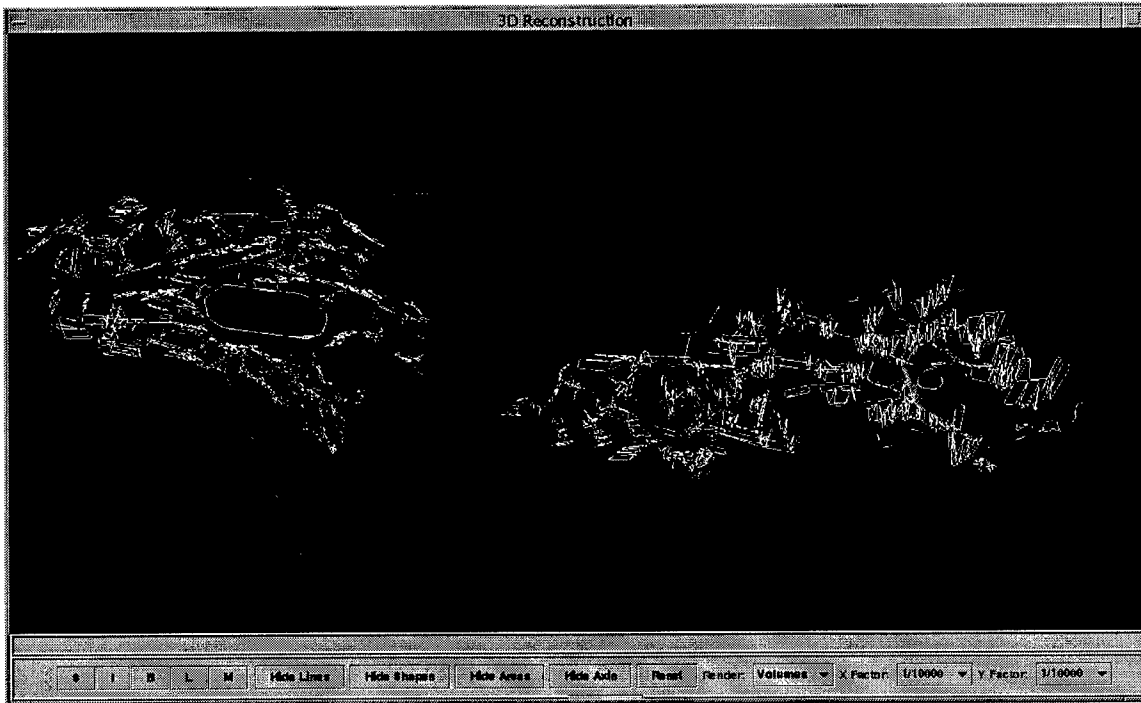
(a)



(b)

Figure 8. Tissue annotation.

Two consecutive H&E (a) and Evan's Blue (b) stained sections of the two mouse mammary glands using to test the system. Ducts are connected within and between sections. Lymph node contours are drawn and also connected between consecutive sections. Connections between sections are shown by changing line color of all connected components every time one of them is visited (selected or just traveled over by the mouse pointer).




(a)



(b)

Figure 9. Tissue Reconstruction.

3D reconstruction of the manually annotated cases used to test our system: (a) H&E stained. (b) Case stained with Evan's Blue. Lymph node volumes have been surface-



rendered to show their three-dimensional shapes. Ducts are identified by spheres and connected by lines within and between sections. The 3D reconstruction is fully interactive. It can be rotated, zoomed, and all the elements are active, in that by clicking on them one can retrieve information about the element, display the original image that contains the element or automatically move the microscope to the selected point.